N-231

Changes in Bacterial Community Structure Correlate with *in situ* Uranium Immobilization

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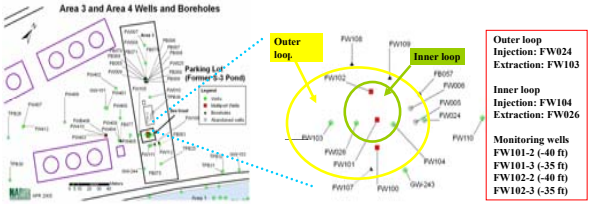
ABSTRACT. Former radionuclide waste ponds at the ERSP-FRC in Oak Ridge, TN, pose challenges for U(VI) bioremediation. The site is marked by acidic conditions, high concentrations of NO_3, chlorinated solvents, and heavy metals. A series of recirculating wells establish a subsurface bioreactor to stimulate microbial growth for *in situ* U(VI) immobilization. Well FW-104 is the injection well for the electron donor (ethanol); well FW-026 is the extraction well for the recirculation loop; well FW-101 is the center of biostimulation; and FW-024 and FW-103 are upstream and downstream wells, respectively. Bacterial community composition and structure of the groundwater from the wells were analyzed via clonal libraries of partial SSU rRNA gene sequences over time. LIBSHUFF analyses for the clonal libraries from FW-104 and FW-101 showed that bacterial communities of the two wells were initially similar but developed changes through time in parallel. The two wells had reduced diversity at high levels of NO_3 and U(VI) with comparable population composition. FW-101 had increased diversity at intermediate levels of NO_3 and U(VI), but diversity was reduced upon NO_3 and U(VI) reduction. This data supported an intermediate-disturbance theory for perturbation effects on the community. Diversity continued to increase in FW-104. LIBSHUFF analysis for the clonal libraries of the five wells on day 535 showed that the bacterial communities of the two wells (FW-101 and FW-026) immediately downstream from the injection point were more similar to the injection well than the outer-loop wells. Diversity indices on day 535 showed that the upstream and injection wells had reduced diversity; whereas the treatment zone and the immediate downstream well both had increased diversity. The furthest downstream well had the lowest diversity compared to other wells. The results indicated that the bacterial community composition and structure changed upon stimulating for metal-reducing conditions, and that sequences representative of the metal-reducers *Ferribacterium*, *Desulfovibrio* spp. and *Anaeromyxobacter* were detected in wells that displayed a decline in both NO_3 and U(VI).

INTRODUCTION. Uranium is a major groundwater contaminant at the U.S. Department of Energy (DOE) Environmental Remediation Sciences Program (ERSP) Field Research Center (FRC) site in Oak Ridge, Tennessee. The sites are also characterized by acidic conditions (pH 3.5), high concentrations of nitrate (up to 160-200 mM), various heavy metals, and other contaminants. A two-phased approach is currently being used at the FRC to deal with these conditions. The first phase includes neutralization of the groundwater pH and aboveground removal of nitrate, chlorinated solvents, calcium and aluminum. The second phase involves recirculation of groundwater supplemented with electron donor to stimulate microbial activities for the denitrification of residual nitrate and uranium reduction *in situ*. A series of re-circulating wells establish subsurface *in situ* U(VI) immobilization. Well FW-104 is the injection well for the electron donor (ethanol); well FW-026 is the extraction well for the recirculation loop; well FW-101 is the center of biostimulation; and FW-024 and FW-103 are upstream and downstream wells for hydraulic flow control, respectively. The purpose of this study is to evaluate the changes in microbial community composition as conditions are stimulated for uranium bio-reduction in the subsurface.



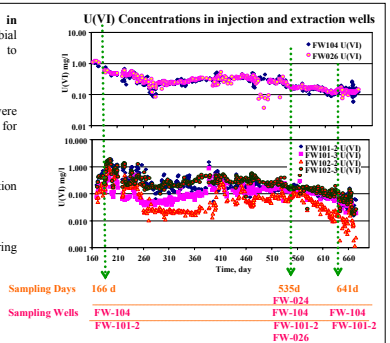
MATERIALS and METHODS

PCR amplification and cloning. The SSU rRNA genes were amplified in a PTC-200 Peltier Thermal Cycler (MJ Research) with the primer pair FD1 and 1540R. The PCR reaction (25.0 µl) contained 12.5 µl BioMix (Bioline, Randolph, MA), 1.0 µl each primer, 9.5 µlsterilized Milli-Q water, 1.0 µl purified DNA (5-10 ng). To minimize PCR-induced artifacts, the optimal number of cycles was determined and five PCR reactions were combined prior to cloning. An aliquot of 5.0 µlof PCR product was run in a 0.8% agarose TAE gel stained with ethidium bromide to evaluate the quality of the amplified fragment.

The PCR products of the SSU rRNA genes were purified using a Promega Kit (Promega, Madison, WI). The purified fragments were cloned using the vector PCR 2.1-TOPO and *Escherichia coli* DH5αTM-T1R competent cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The cloned inserts were amplified with vector-specific primers, M13 forward and M13 reverse. The resulting amplification products were analyzed as described above.

Sequence and phylogenetic analysis. PCR products (20.0 µl) amplified with vector-specific primers were purified with a Montage PCRu96 plate according to manufacturer's instructions (Millipore, Bedford, MA). DNA sequences were determined with a BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions at 1:4 dilution using internal sequencing primers. Sequence reaction products were run on the ABI model 3730 DNA sequencer (Applied Biosystems). DNA sequences were assembled and edited using the SequencherTM program (v. 4.0, Gene Codes Corporation, Ann Arbor, MI).

Figure 1. U(VI) concentrations in injection and extraction wells. Microbial samples were analyzed according to uranium level change at different days.

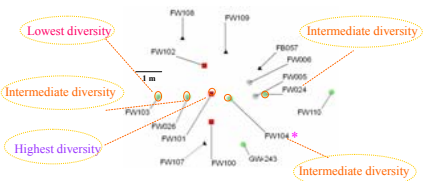


Day 9-136 Clean Water Flush
Nitrate, aluminum, and calcium were removed to favorable levels for bioremediation

Day 137-184 in situ Denitrification
Nitrate was further reduced by stimulation of denitrification by adding ethanol

Day 185-712 in situ U(VI) Reduction
Uranium concentrations in monitoring wells were reduced to below 30 µg/L.

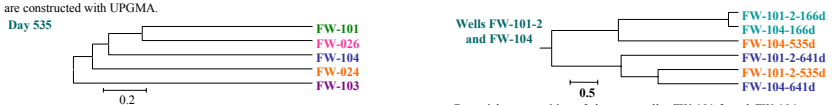
Figure 2. Diversity indices of the circulating wells on day 535. The upstream and injection wells (FW-024 and FW-104) had reduced diversity; whereas the treatment zone (FW-101-2) and the immediate downstream well (FW-026) both had increased diversity. The furthest downstream well (FW-103) had the lowest diversity compared to other wells.



* Denotes injection well. Ethanol is the electron donor.

Operational taxonomic units (OTI): based on unique, partial SSU rRNA gene sequences (≤97%)
Reciprocal of Simpson's index (1/D): higher number represents more diversity
Evennes (E): As Evenness approaches 1, the population is more evenly distributed

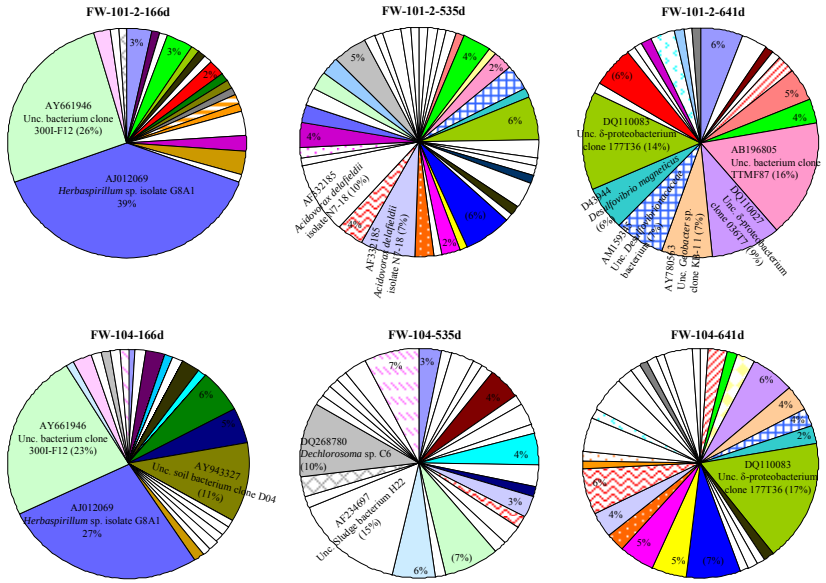
Figure 3. SSU rDNA clonal libraries. The changes in community structure was scored based on correlated distances used for LIBSHUFF analysis and the trees are constructed with UPGMA.



On day 535 showed that the bacterial communities of the two wells, FW-101 and FW-026, immediately downstream from the injection point were more similar to the injection well than the outer-loop wells, FW-024 and FW-103.

Bacterial communities of the two wells, FW-101-2 and FW-104 were initially similar but developed changes over time. Relationships suggested that similar changes occurred at both sites, but the injection well community lagged in changes.

Figure 4. The clone distribution and overlap among time points for the subsurface SSU rRNA gene sequences in FW104 (injection well) and FW-101-2 (center of biostimulation) on days 166, 535, and 641. Each color represents a unique sequence, and the same between sites represents the same OTU. Pie segments depicted as white represent unique sequences observed only at the respective time point. The sequence names are denoted for predominant clones and the respective percentage for that time point. At earlier time points, the subsurface was predominated by denitrifying bacteria and typical soil bacteria. Upon biostimulation, sulfate and metal-reducers such as *Desulfovibrio*-like sequences and *Geobacter*-like sequences were observed.



- AM159293 Unc. *Actinobacteriaceae* clone Hrh678
- AJ888538 Unc. *Actinobacterium* clone TH417
- DQ316356 Unc. *Actinobacterium* clone ST11-10
- AY568514 *Burkholderia* sp. SE-10
- AB091199 *Frateuria aurantia* strain:IFO13330
- AY053477 Unc. Bacterium clone AT425_Eu648
- AY239581 Unc. bacterium clone CARB_ER2_13
- DQ066996 Uncultured bacterium clone pLW-58
- AY635987 Unc. *Phyllobacterium* sp. clone Ph
- AB195161 *Acaligenes defragans* strain:PD-19
- AF345552 *Rhizobium* sp. SDW058
- DQ177525 *Sphingomonas* sp. JQL4-5
- AB195755 Aquatic bacterium clone R1-B23
- AJ412676 Uncultured eubacterium clone 92
- AF235994 Unc. α-proteobacterium F0917
- AY661958 Unc. bacterium clone 010A-D01
- DQ401253 *Frateuria* sp. iMSN36
- AY662006 Unc. Bacterium clone 015B-B03
- AY568503 *Afipia broomeae*
- AY037569Unc. soil bacterium clone S022
- M34417 *Desulfotomaculum orientis*

Figure 4. Clonal library diversity in wells FW-101-2 and FW-104.

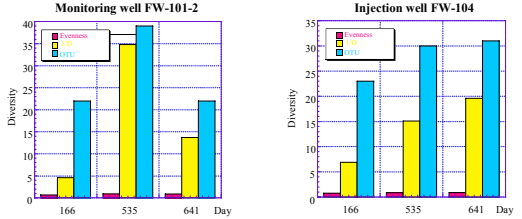


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Table1. pH, uranium, nitrate, and sulfate levels in wells FW-101-2 and FW-104 on sampling days.

	Monitoring well FW-101-2			Injection well FW-104		
Sampling days	**166d**	**535d**	**614d**	**166d**	**535d**	**614d**
pH	6.511	6.352	6.009	6.159	6.875	5.907
U (VI) (µg/L)	0.791	0.186	0.043	1.053	0.174	0.163
NO_3 (mM)	0.202	0	0	0.812	0.038	0.005
SO_4 (mM)	0	0.07	0.33	0	0	0.04

The two wells had reduced diversity at high levels of NO_3 and U(VI) with comparable population composition. FW-101 had increased diversity at intermediate levels of NO_3 and U(VI), but diversity was reduced upon NO_3, U(VI) and SO_4 reduction. Diversity continued to increase in FW-104.

Figure 6. Phylogenetic relationships of unique cloned SSU rRNA gene sequences of observed sulfate and metal-reducers from the subsurface and reference sequences from GenBank classified as *Desulfovibrio* sp., *Geobacter* sp., and *Anaeromyxobacter* sp. The tree is based on neighbor-joining method and pairwise deletion constructed in MEGA (version 2.1; http://megasoftware.net). The accession numbers for reference sequence are listed in the tree (e.g. AF053751 *Desulfovibrio alcoholvorans).*

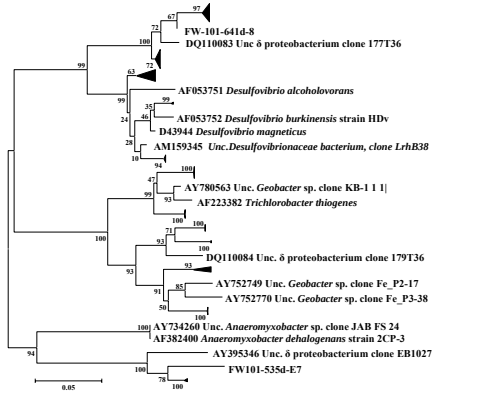

Table 2. Observed predominant clones from each clonal library of wells FW-101-2 and FW-104 at sampling days.

	Monitoring well FW-101-2	Injection well FW-104
166d	Unc. bacterium clone 300I-F12 (26%) *Herbaspirillum* sp. isolate G8A1 (39%)	Unc. bacterium clone 300I-F12 (23%) *Herbaspirillum* sp. isolate G8A1 (27%) Unc. soil bacterium clone D04 (11%)
535d	*Acidovorax delafieldii* isolate N7-18 (10%) *Acidovorax delafieldii* isolate N7-18 (7%) Unc. δ-proteobacterium clone 177T36 (6%) Unc. *Actinobacteriaceae* clone Hrh678 (6%) *Dechlorosoma* sp. C6 (5%)	Unc. Sludge bacterium H22 (15%) Unc. bacterium clone 300I-F12 (7%) Unc. Bacterium clone 015B-B03 (7%) *Acaligenes defragans* strain:PD-19 (6%) *Dechlorosoma* sp. C6 (10%)
641d	Unc. bacterium clone TTMF87 (16%) Unc. δ-proteobacterium clone 177T36 (14%) Unc. *Desulfovibrionaceae* bacterium (7%) *Desulfovibrio magneticus* (6%) Unc. δ-proteobacterium clone 036T7 (9%) Unc. *Geobacter* sp. clone KB-1 1 (7%) Unc. *Phyllobacterium* sp. clone Ph (6%) *Rhizobium* sp. SDW058 (6%)	Unc. δ-proteobacterium clone 177T36 (17%) Unc. *Desulfovibrionaceae* bacterium (4%) *Desulfovibrio magneticus* (2%) Unc. *Actinobacteriaceae* clone Hrh678 (7%) Unc. δ-proteobacterium clone 036T7 (6%) Unc. *Geobacter* sp. clone KB-1 1 (4%)

SUMMARY

- In *situ* bioreduction of U(VI) was stimulated by injection of ethanol.
- Microbial diversity increased post-stimulation.
- On day 535d, microbial community composition was different along the gradients.
- Sulfate and metal reducers were detected at the site post-stimulation.